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Essential role of the HMG domain in the function of yeast mitochondrial histone HM: Functional complementation of HM by the nuclear nonhistone protein NHP6A

(HMG protein/*Saccharomyces cerevisiae*/DNA supercoiling/petite)

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ABSTRACT **The yeast mitochondrial histone protein HM is required for maintenance of the mitochondrial genome, and disruption of the gene encoding HM (*HIM1/ABF2*) results in formation of a respiration-deficient petite mutant phenotype. HM contains two homologous regions, which share sequence similarity with the eukaryotic nuclear nonhistone protein, HMG-1. Experiments with various deletion mutants of HM show that a single HMG domain of HM is functional and can restore respiration competency to cells that lack HM protein (*him1* mutant cells). The gene encoding the putative yeast nuclear HMG-1 homolog, the NHP6A protein, can functionally complement the *him1* mutation. These results suggest that the HMG domain is the basic unit for the function of HM in mitochondria and that the function of HMG-1 proteins in the nucleus and HM in the mitochondrion may be equivalent.**

The compaction of nuclear DNA is achieved at the first level by formation of the nucleosomes in which DNA is wrapped twice around the octameric complex of histones (1). In bacteria there is a histone-like protein named HU, which is an abundant DNA-binding protein capable of introducing negative supercoil turns into relaxed circular DNA *in vitro* (2, 3). HU also forms a beaded nucleosome-like structure with DNA (2, 4). The functional homology between HM and HU *in vivo* (5) suggests that the mitochondrial DNA may also be assembled into a nucleosome-like structure in mitochondria. Caron *et al.* (6) first purified and characterized the yeast mitochondrial histone-like protein HM and showed that it is present in approximately the same mass as the mitochondrial DNA and is able to introduce supercoil turns into circular relaxed DNA. They suggested that HM may play a structural role in organization of the mitochondrial genome (6). We purified the HM protein and cloned the gene and designated it *HIM1* (5). The gene encodes the same protein as the yeast DNA-binding protein ABF2, which was purified on the basis of its higher binding affinity for a nuclear autonomous-replicating sequence than for nonspecific DNA sequences (7). Disruption of the *HIM1* (*ABF2*) gene in yeast leads to the loss of mitochondrial DNA and produces a respiration-deficient petite phenotype (5, 7). HM (ABF2) is encoded by a nuclear gene and contains two domains that have significant sequence similarity to a region of the eukaryotic nuclear nonhistone protein HMG-1 (5, 7). Parisi and Clayton (8) reported a human mitochondrial transcription factor (mtTF1) that also has two HMG domains. Like HM, mtTF1 introduces negative supercoils into relaxed circular DNA *in vitro* (5, 6, 9, 10). The *Xenopus laevis* mitochondria also contain a DNA-binding protein (mtDBP-C), which supercoils DNA (11), and the N-terminal sequence has homology to HMG-1 proteins (12). Therefore, it appears that the mitochondria of different species have a similar HMG-1-like protein.

HMG-1 proteins are abundant nonhistone proteins in the cell nucleus (13). However, their cellular functions remain poorly defined (13). The primary sequence of HMG-1 proteins is well conserved between species and usually consists of two HMG domains and a C-terminal acidic tail domain (13). The putative yeast HMG-1 proteins NHP6A and NHP6B contain one HMG box and no acidic tail domain (14). Several transcription factors containing HMG domains have recently been discovered and indicate a widespread use of this DNA-binding domain among proteins for DNA binding and bending (15).

In this report, we investigated the role of the HMG domains of HM *in vitro* and in yeast mitochondria by producing various deletion mutants of HM. Here we report that each of the two HMG domains of HM is functional *in vivo*. In support of the major role of the HMG domain of HM, we found that the *NHP6A* gene encoding the putative yeast nuclear HMG-1 protein can complement the *him1* mutation. The HM and HMG-1 proteins are probably evolved from a common ancestral gene and may serve common functions in the mitochondrion and the nucleus, respectively.

MATERIALS AND METHODS

***Saccharomyces cerevisiae* Strains.** The generation of the *him1* mutant strain TM144-15d (*MATa him1::URA3 ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112*) and wild-type strain TM144-15a (*HIM1*) was described (5). The strains created by transformation of TM144-15d with plasmids are as follows: TM164 (pRS314G), TM165 (pGal-HIM1), TM166 (pGal-HIM1ΔC), KLR11 (pGal-HIM1ΔN), KLR12 (pGal-HIM1Δ1–27), KLR13 (pGal-HIM1ΔCΔ1–27), KLR15 (pGal-HIM1ΔCΔ17–27), KLR16 (pGal-mNHP6A), and KLR40 (pGAL-nNHP6A).

Plasmid Construction. For expression of the mature form of HM and deletion mutants in *Escherichia coli*, the appropriate *HIM1* sequences were amplified from yeast genomic DNA by PCR or recombinant PCR (16) and ligated into the *Stu* I site downstream of the maltose-binding protein gene (*malE*) in the plasmid pMALc (New England Biolabs). For expression in yeast, *HIM1* and deletion mutants were fused to the *HIM1* presequence by recombinant PCR and inserted in the *Cla* I site downstream of the *GAL10* promoter in a yeast shuttle vector pRS314G (*TRP1, CEN6, ARS4*; see ref. 5). For expression of NHP6A in *E. coli*, the *NHP6A* gene (14) was obtained by amplification of yeast genomic DNA by PCR and ligated into pMALc. For targeting NHP6A to the mitochondria, the *HIM1* presequence (5, 7) was fused with the *NHP6A* gene before inserting into pRS314G. We also constructed a plasmid containing the *NHP6A* gene without the *HIM1*

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Abbreviation: MBP, maltose-binding protein.

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presequence (pGAL-nNHP6A). All DNA sequences were verified by the dideoxynucleotide chain-termination sequencing method (17).

Expression of the Mutant HM Proteins and NHP6A in *E. coli*. HM, mutant HM proteins, and NHP6A were expressed as fusion proteins with maltose-binding protein (MBP) in *E. coli* strain XL1-Blue and purified by affinity chromatography on an amylose column according to a protocol provided by New England Biolabs. The fusion proteins were cleaved with Factor Xa, and HM (or NHP6A) was further purified by chromatography on a Bio-Rex 70 (Bio-Rad) column. The purity of the proteins was confirmed by SDS/PAGE. Most of the proteins produced a single band on the gel; some, however, also contained some MBP protein. MBP, however, does not bind to DNA or interfere with the binding of HM to DNA (data not shown). The concentration of HM was determined by acid turbidity assay: 40 μl of protein solution and 40 μl of 50% trichloroacetic acid were mixed and incubated at room temperature for 5 min; the turbidity formed was determined at 400 nm and compared with those obtained with known amounts of calf thymus histone H1.

DNA Mobility-Shift Assay. The reaction mixture (20 μl) included various amounts of HM or the mutant proteins and 5 fmol of ^{32}P-labeled double-stranded DNA fragment (the sequence is AATTCGAGCTCGGTACCCCACTGCCCAGTCAAGTGTTCCTTGAACAGTAGGGATCCT) in 10 mM Tris·HCl, pH 7.5/50 mM NaCl/10% (vol/vol) glycerol/1 mM EDTA/1 mM dithiothreitol. This DNA sequence contains 30 bp of a rat thyroglobulin promotor sequence flanked by 27 bp of pUC18 polylinker sequence and was used here because it was conveniently available in our laboratory and not for any reason pertaining to its origins. It has been shown that HM (ABF2) binds to DNA nonspecifically (9). The mixture was incubated at room temperature for 30 min and loaded onto a 6% polyacrylamide gel buffered with 22.5 mM Tris base/22.5 mM boric acid/0.625 mM EDTA. After electrophoresis, the gel was dried and exposed to an x-ray film.

DNA Supercoiling Assay. Each incubation mixture (20 μl) contained 200 ng of pBR325 plasmid DNA and various amounts of HM or the mutant proteins and 2 units of topoisomerase I in 10 mM Tris·HCl, pH 8.0/1 mM EDTA/100 mM NaCl and incubated at 37°C for 30 min and then stopped by addition of 5 μl of 1% SDS/0.25% bromophenol blue/25% glycerol. DNA was then analyzed by electrophoresis on 1% agarose gel in 40 mM Tris acetate/2 mM EDTA.

Restoration of Respiration Competency of *him1* Mutant Cells by Mutant HM Proteins. Yeast plasmids containing the *HIM1* gene or a *HIM1* mutant gene were introduced into the *him1* mutant strain TM144-15d using the LiCl method modified from Ito *et al.* (18). The transformants were inoculated into medium containing 2% glucose or 0.5% galactose plus 1.5% raffinose and grown until late logarithmic phase at 30°C. The cells were plated onto YPGalGE or YPDGE plates (1% yeast extract/2% Bactopeptone/3% glycerol/2% ethanol/0.1% galactose for YPGalGE or 0.1% glucose for YPDGE) (5, 19) and incubated at 37°C (5).

Preparation of Mitochondria. All yeast strains transformed with plasmid were grown in synthetic complete (SC) medium (without tryptophan and uracil) containing 2% galactose or 2% glucose (19) until stationary phase at 30°C and harvested by centrifugation at 3600 × *g* (Sorvall SA600 rotor). The spheroplasts were prepared by incubation with Zymolyase-20T (0.625 mg/ml) in S buffer (1 M sorbitol/10 mM Pipes, pH 6.5) and washed in S buffer and then suspended in 250 mM sucrose/1 mM EDTA/10 mM Pipes, pH 6.5, and homogenized in a tight-fitting Dounce homogenizer. The cell debris and nuclei were removed by centrifugation at 1500 × *g* for 5 min. The mitochondria were pelleted by centrifugation at 10,000 × *g* for 10 min. The differential centrifugation was repeated once. The mitochondrial preparations were then used for analysis of proteins by Western gel blotting without further treatment.

Western Immunoblotting. The mitochondrial proteins fractionated by SDS/PAGE were transferred to a nitrocellulose membrane by electroblotting, and the membrane was probed with polyclonal rabbit antibodies for HM protein (5) and subsequent incubation with anti-rabbit IgG conjugated with alkaline phosphatase according to a published protocol (20).

RESULTS

Construction of Deletion Mutants of HM Protein. Fig. 1 shows a schematic diagram of the domain structure of the HM, HMG-1, and NHP6A proteins. The mature form of HM is 157 amino acids long (7). The two HMG domains in HM reside between amino acid positions 17 and 84 and between positions 90 and 157. The HMG domain may be considered to consist of two subdomains, one region predicted to have a β-sheet structure and a second subdomain consisting of two α-helices (21). An 11-amino acid stretch in the first subdomain is especially well conserved among HMG domain-containing proteins and may be a DNA-binding motif (22). The amino acid positions of these subdomains within the protein sequences of HM, HMG-1, and NHP6A are shown in Fig. 1. To investigate the role of each HMG domain as well as the subdomains in HM, we constructed various deletion mutants of HM as summarized in Fig. 2. To obtain the mutant proteins for DNA-binding assays, the normal and mutant *HIM1* genes without the HM presequence were fused to the *E. coli* gene encoding MBP in an expression vector (pMALc). The fusion proteins produced in *E. coli* were affinity purified on an amylose column, and the MBP and HM proteins were separated by cleavage with Factor Xa. HM was further purified by chromatography on a Bio-Rex 70 column. The purity of HM and HM mutant proteins was confirmed by gel electrophoresis.

DNA-Binding Activity of Mutant HM Proteins. To study the DNA-binding activity of HM proteins, we used the DNA mobility-shift assay, which allows separation of DNA–

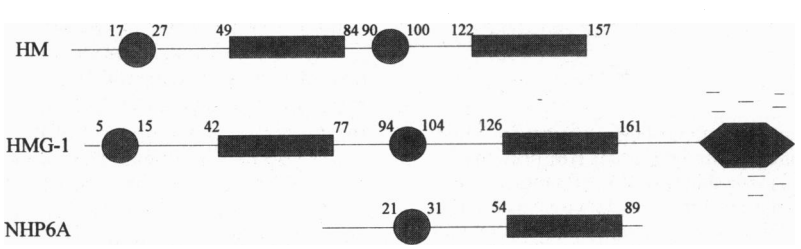

FIG. 1. Schematic comparison of the domain structure of HM, HMG-1, and NHP6A proteins. Circle motifs correspond to a well-conserved 11-amino acid stretch within the HMG box; rectangle motifs correspond to the two tandem α-helix subdomains (21, 22). The hexagonal motif with the negative signs around it (HMG-1) corresponds to the acidic domain of HMG-1 (13). Numbers correspond to amino acid positions bordering each subdomain. Subdomains in the HMG box have not been designated based on a functional identification, but rather on the basis of amino acid conservation in these regions and protein structural predictions.

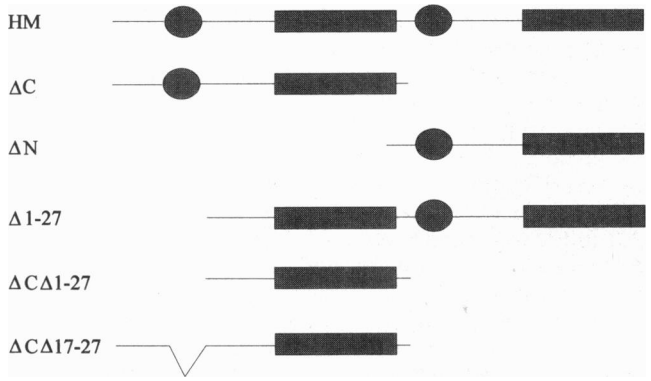

FIG. 2. Structure of mutant HM proteins. HM deletion mutants retained the following amino acid regions: ΔC (1–89), ΔN (82–157), Δ1–27 (28–157), ΔCΔ1–27 (28–89), ΔCΔ17–27 (1–16 and 28–89). Numbers correspond to amino acid positions in the mature protein (not the preprotein).

protein complexes from free DNA probe on nondenatured polyacrylamide gels (23). HM binds to a short (57 bp) DNA fragment and forms multiple DNA–protein complexes (Fig. 3*A*). Similar observations have been made with the *E. coli* histone-like protein HU binding to short DNA fragments (24). The first half molecule of the protein (HMΔC), containing one intact HMG domain, can bind to DNA and forms multiple DNA–protein complexes as HM does (Fig. 3*A*). The complexes formed with HMΔC migrate faster than those formed with HM due to its smaller size (9.3 vs. 18.6 kDa). The formation of multiple complexes indicates that several molecules of either HM or HMΔC can bind to each DNA molecule. Protein crosslinking experiments showed that HM (and HMΔC) forms multimers *in vitro* (data not shown). All of the other mutant HM proteins showed reduced DNA-binding activity (Fig. 3*A*).

DNA Supercoiling Activity of Mutant HM Proteins. It has been shown that binding of DNA to the octameric complex of four core histones H2A, H2B, H3, and H4 at physiological ionic strength results in the introduction of supercoil turns into relaxed circular DNA, and the number of supercoil turns introduced is roughly equal to that of nucleosomes formed (25). The *E. coli* histone-like protein HU (2, 3) and yeast mitochondrial histone HM (5, 6, 9, 10) also introduce negative supercoil turns into relaxed DNA. We have used this system to assay for the introduction of supercoiled turns into relaxed plasmid DNA by mutant HM proteins. HM converts relaxed plasmid DNA into the supercoiled form maximally at a protein/DNA ratio of 1 (ref. 6; Fig. 3*B*). HMΔC, which contains the first HMG domain in HM, can also introduce supercoils into relaxed plasmid DNA and does so maximally at a protein/DNA ratio of 1 (Fig. 3*B*). The mutant that retains only the second HMG domain (ΔN; Fig. 3*B*) and all the other mutant HM proteins (data not shown) did not have DNA-supercoiling activity *in vitro*.

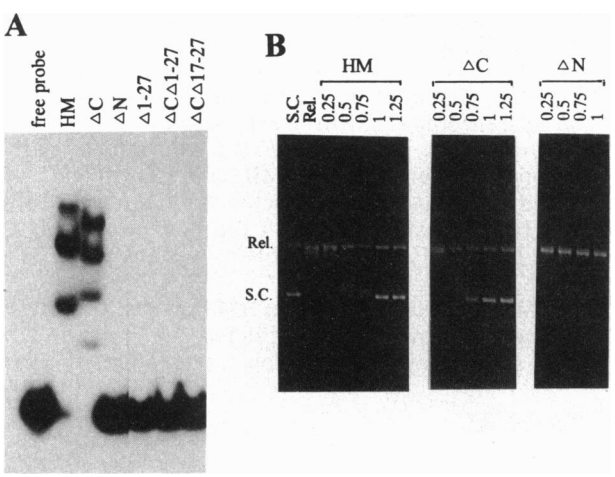

FIG. 3. DNA-binding activity of mutant HM proteins. (*A*) DNA mobility-shift assay. Recombinant HM and mutant HM proteins (20 ng) purified from *E. coli* were incubated with ^{32}P-labeled DNA fragment and applied on nondenatured polyacrylamide gel for electrophoresis as described. (*B*) DNA supercoiling activity of mutant HM proteins. Relaxed form of plasmid pBR325 (200 ng) was mixed with increasing amounts of HM or mutant HM proteins (mass ratio shown above each lane) in the presence of topoisomerase I as described. After incubation, the reaction mixture was applied on agarose gel and electrophoresed. S.C., supercoiled form; Rel., relaxed form.

Restoration of Respiration Competency of *him1* Mutant Cells by Mutant HM Proteins *in Vivo*. Growth of *him1* mutant cells in the presence of a nonfermentable carbon source such as glycerol, where a functional mitochondrial genome is essential, is slower than wild-type cells at 30°C and completely blocked at 37°C (5). In the presence of fermentable sugars such as glucose or galactose, where the function of the mitochondrial genome is dispensable, the growth of *him1* cells is unaffected at either temperature. Therefore, we investigated the ability of the HM mutant proteins to restore the growth of *him1* mutant cells on glycerol at 37°C. The *HIM1* gene and the mutant *HIM1* genes were fused to the *GAL10* promoter in a yeast shuttle vector (pRS314G) and all genes contained the *HIM1* presequence to allow mitochondrial localization. These constructs permit production of the HM proteins in the presence of galactose but not in the presence of glucose. These plasmids were introduced into the yeast strain TM144-15d (the *him1* mutant) and assayed for their ability to restore respiration competency of the *him1* mutant cells. The transformants were grown in medium containing galactose or glucose until logarithmic phase. The cells were then streaked on glycerol plates, which contained a low concentration (0.1%) of either galactose (YPGalGE) or glucose (YPDGE), and grown at 37°C. Fig. 4 (*Upper*) demonstrates that the *him1* mutant cells containing the plasmids encoding HM (pGAL-HIM1), the first HMG box of HM (pGAL-HIM1ΔC), or the second HMG box of HM (pGAL-HIM1ΔN) are dependent on a small amount of galactose to be respiration competent, whereas the vector used in plasmid construction cannot confer respiration competency to the mutant cells. The *HIM1* wild-type strain can grow on either medium. Due to the necessity of addition of galactose or glucose for the induction or repression of the *GAL10* promoter, respectively, there was a limited growth of the *him1* mutant cells on either plate. These results show that the mutant HM proteins containing an intact HMG domain (HMΔC or HMΔN) is functional in mitochondria. On the other hand, an HMG domain lacking the 11-amino acid putative DNA-binding subdomain (mutant ΔCΔ1–27 in Fig. 4 *Lower* or ΔCΔ17–27; data not shown) was not functional *in vivo*. However, HMΔ1–27, which lacks this subdomain but has an intact second HMG box, is functional *in vivo* (Fig. 4 *Lower*). Western immunoblotting experiments with antibody to HM protein confirmed that all of the mutant HM proteins were present in yeast mitochondria when cells were grown in the presence of galactose but not glucose (data not shown).

Functional Complementation of *him1* Mutant Cells by the Yeast *NHP6A* Gene. As shown in Fig. 1, the yeast nuclear nonhistone protein NHP6A has one HMG domain that shares significant sequence similarity (45%) with the HMG domain of bovine HMG-1 protein (14). Therefore, we were interested to determine whether NHP6A can substitute for HM in mitochondria. First, we investigated the ability of the NHP6A protein to supercoil DNA *in vitro*. The NHP6A protein produced in *E. coli* (Fig. 5*A*), like HM, supercoiled

FIG. 4. Restoration of respiration competency of *him1* mutant cells by mutant HM proteins. The *him1* mutant yeast cells were transformed with the yeast plasmid carrying *HIM1* or mutant *HIM1* genes (with the *HIM1* presequence) fused with the *GAL10* promoter. Cells were grown in medium containing either glucose or galactose until logarithmic phase and then plated onto glycerol plates containing a small amount (0.1%) of either glucose (YPDGE) or galactose (YPGalGE) and incubated at 37°C. HIM1 represents wild-type cells (TM144-15a), and all the other strains represented in this figure are *him1* cells carrying various mutant *HIM1* genes (see Fig. 2 for the designation).

DNA maximally at a protein/DNA mass ratio of ≈1 (Fig. 5*B*). For expression in mitochondria, the *NHP6A* gene was fused with the DNA sequence corresponding to the HM presequence, the presumed mitochondrial localization signal (5, 7), and placed behind the *GAL10* promoter (pGal-mNHP6A). The *him1* mutant cells containing pGal-mNHP6A grew as well as the mutant cells containing pGal-HIM1 on YPGalGE but grew poorly on YPDGE (Fig. 5*C*). The *NHP6A* gene fused to the *GAL10* promoter without the *HIM1* presequences fused to it (pGAL-nNHP6A) did not complement the *him1* mutant cells (Fig. 5*C*). This result demonstrates that NHP6A can substitute for HM in yeast mitochondria, converting respiration-deficient *him1* cells to respiration-competent cells.

DISCUSSION

In this report, we have shown that either one of the two HMG domains of the yeast mitochondrial histone HM is functional in mitochondria, being capable of restoring respiration competency to the *him1* mutant cells. We also conclude that the conserved 11-amino acid putative DNA-binding motif is an essential component for the function of the HMG box of HM *in vivo*. The yeast nuclear nonhistone protein NHP6A, which has significant sequence similarity to HMG-1 proteins and is the putative yeast HMG-1 homologue, can also complement the absence of HM *in vivo* when the protein has the presumed HM presequence to target its import to the mitochondrion. Our attempt to produce human HMG-1 in yeast mitochondria was unsuccessful due to the instability of the DNA sequence during cloning and expression (data not shown).

It is not clear why the second HMG domain (ΔN) and the mutant protein lacking the first 27 amino acids but containing the intact second HMG domain (Δ1–27) can complement HM *in vivo* but have neither DNA-binding nor DNA-supercoiling activity *in vitro*. It is possible that correct folding of these mutant HM proteins is facilitated in yeast cells but not when they are overproduced in *E. coli*. This notion is supported by the inefficient cleavage of the MBP-HMΔN fusion protein by the factor Xa protease *in vitro*. In this sense, the *in vivo* assay system described in this report probably provides a more realistic assessment of the protein regions important for the function of HM in mitochondria.

The role that HM or a half molecule of HM plays in maintaining the mitochondrial genome is presently unclear. The DNA supercoiling activity and the protein–protein interactions of HM may be involved in compaction as well as maintenance of DNA in mitochondria. It will be important to investigate whether the HM protein forms multimeric complexes or nucleosome-like structures on DNA as does the *E. coli* HU protein, which is functionally homologous to HM (5). It is also possible that HM is not the only protein required for maintenance of the mitochondrial genome since the *him1* mutant cells containing a functional mitochondrial genome can be maintained in the presence of glycerol at 30°C (5). Recently, the *PIF1* gene, a helicase-encoding gene, was reported to be essential for maintenance of mitochondrial DNA at 36°C and the *pif1* null mutants would not grow on glycerol at this elevated temperature (26). These phenotypes are also associated with *him1* mutant (5) and, considering the topological effects that both proteins exert on DNA, may indicate a relationship between PIF1 and HM proteins in maintaining the mitochondrial DNA.

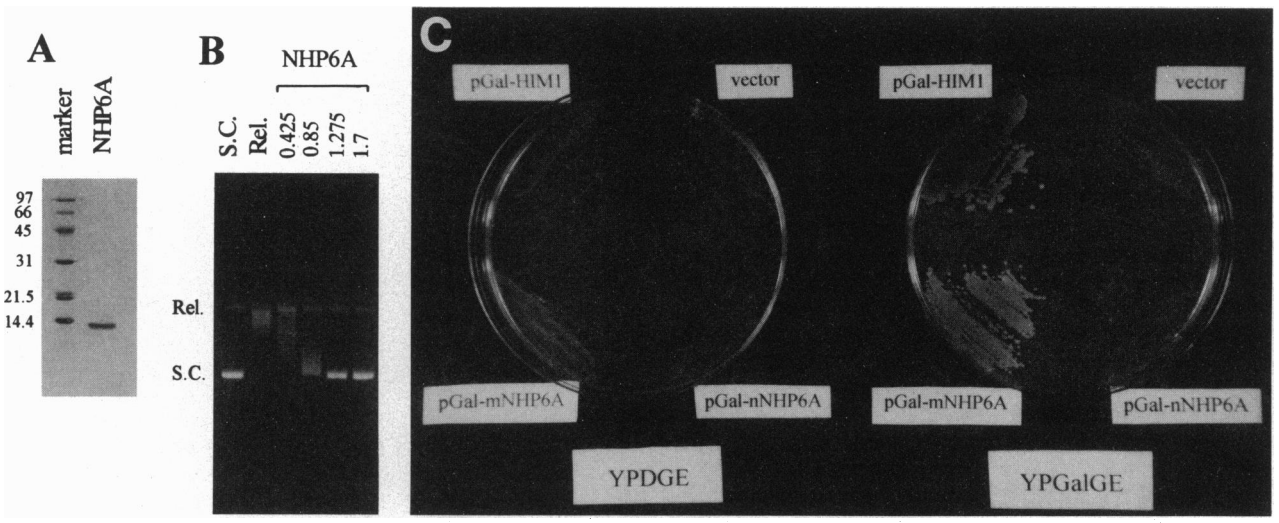

FIG. 5. DNA supercoiling activity of NHP6A protein and complementation of *him1* mutant. (*A*) Coomassie blue staining of purified NHP6A protein after SDS/PAGE. Left lane, size markers (kDa); right lane, NHP6A. NHP6A protein was purified from *E. coli* cells expressing the *NHP6A* gene in plasmid pMALc. (*B*) DNA supercoiling activity. Assay was carried out as described in Fig. 3. Mass ratios of NHP6A protein to plasmid DNA are shown above the lanes. (*C*) Complementation of *him1* mutant by *NHP6A*. The *him1* mutant cells were transformed with plasmids carrying the normal *NHP6A* gene (pGAL-nNHP6A) and the *NHP6A* gene fused to the *HIM1* presequence (pGAL-mNHP6A). Assay was carried out as described in Fig. 4.

The fact that the HMG domains of HM and the nuclear nonhistone protein NHP6A can complement HM *in vivo* strongly suggests that HM and the nuclear HMG-1 proteins are derived from a common ancestor gene during eukaryotic evolution. We found that HM and the *E. coli* histone-like protein HU can complement each other either in yeast mitochondria or in *E. coli*, although the two proteins do not share any sequence similarity (5). The HM and HU proteins, however, share common properties: DNA-binding and DNA-supercoiling activity (5). In view of the bacterial origin of mitochondria, it is possible that the original mitochondrial gene encoding an HU-like protein was lost during eukaryotic evolution along with most of the mitochondrial genome, and that an HMG-1-like protein encoded by a nuclear gene replaced the function of an HU-like protein. An alternative evolutionary path may have been taken by the chloroplast, which also has bacterial origins (27). The chloroplast has an abundant histone-like protein that is recognized by antibodies to HU (28).

The ability of the NHP6A protein to functionally complement the loss of HM, as shown in this report, indicates that the role of NHP6A protein in the nucleus may be similar to the role of HM in mitochondria. And the recent finding of functional homology between HM and *E. coli* HU (5) suggests that HM, NHP6A, and HU may all serve similar functions in their respective environments. It will be important to investigate whether all three of these proteins can complement each other in all three environments.

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